Timely Digits?

Mathura Shanmugasundaram and Benoît Robert

Department of Developmental and Stem Cell Biology, *Institut Pasteur and CNRS URA2578, 75015 Paris, France*

Correspondence to Benoît Robert*: Department of Developmental and Stem Cell Biology, Institut Pasteur, 25, rue du Docteur Roux, 75015, Paris. Tel.: +331 45688965. benoit.robert@pasteur.fr*
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A clock is a device to chop up time, a continuous dimension of our universe, into discrete units, thereby allowing us to count hours. Less intuitively, clocks are used by several organisms to chop up continuous embryonic fields into discrete units and, thus, are instrumental in generating shape. Of note, discrete structures are pivotal to life and no complex organism can emerge from continuous, undifferentiated fields. This way, clocks are essential devices for morphogenesis. In this issue of the *Journal of Molecular Biology*, Sheeba *et al.* from Isabel Palmeirim's laboratory in Lisbon review current evidence supporting the involvement of clockwork in the limb to generate the discrete bony structures of digits.

In 1997, Palmeirim *et al.* were the first to uncover the existence of a molecular clock mechanism in chick somitogenesis, which since then has become the most heuristic model for biological clocks in morphogenesis [1]. Very recently, segmentation of the insect *Tribolium* has been shown to depend partially on a vertebrate-like clock [2], suggesting that this mechanism may be general to segmented organisms. Initially, Palmeirim *et al.* demonstrated that PSM (*p*re-*s*omitic *m*esoderm) cells undergo oscillatory expression of the *hairy1* gene (90 min periodicity in the chick) that strikingly matches the period of somite formation. These observations fitted well in the kinematic "Clock and Wavefront model" proposed by Cooke and Zeeman in 1976 to explain somitogenesis [3]. This dynamic model hypothesized the interaction of a positional information gradient down the anterior–posterior axis of vertebrate embryos (*the wavefront*), which is regulated by a molecular oscillator (*the clock*) that sets the time for cells to undergo rapid changes in cell locomotion and adhesiveness, thus resulting in the PSM segmentation into distinct somites. Since then, the somitic clock has been highly refined. Many genes belonging to several signaling pathways, such as Notch, Wnt and FGF, have been shown to be rhythmically expressed in the posterior PSM. It has now become clear that oscillators play a pivotal role in the PSM as a pacemaker validating the temporal periodicity of somitogenesis that in turn is translated spatially to form the periodic boundaries of the somites.

For a clock to tick, feedback mechanisms may be sufficient, and such mechanisms have been unraveled either within a single signaling pathway or across several pathways interacting with each other [4]. The somitic clock has been shown to depend on intrinsic, cell autonomous mechanisms and cell interactions [5]. A biological clock also requires a GO and a STOP signal. Such signals are still to be defined for the somitic clock, which starts ticking during gastrulation, long before somitogenesis starts, and stops when somites reach the end of the PSM, leaving no cells to be further incorporated into somites. A clock further must be linked to some other mechanism to elicit action and be more than an object of wonder: a switch to automatically start your dishwasher at night, an organized human society to translate the Angelus bell ringing into division of the working day in medieval Europe. According to Cooke and Zeeman, this is where the wavefront intervenes. For somitogenesis, the Wnt pathway appears critical in linking the clock and the wavefront [6]. Despite some pending questions, it is now beyond doubt that a clock is causal in the formation of somites.

Limbs also are particularly relevant to a clock mechanism. AER (*a*pical *e*ctodermal *r*idge) extirpation experiments performed by John Saunders in 1948 first linked time with morphogenesis in limb development. The earlier the removal of the AER, the more severe the truncation; only proximal structures (e.g., humerus) develop. When the AER is removed later, more distal chick limb structures form, and even all but the most distal phalanx of digit 3, if the experiment is performed

late enough [7,8]. From these results, models that link time and limb morphogenesis were derived. Lewis Wolpert and his collaborators proposed that positional information along the PD axis of the limb is specified by the length of time undifferentiated mesenchymal cells spend, or the number of divisions they undergo, in the area underneath the AER (an area adequately called the "progress zone") [8]. Decades later, the model was refined by showing that PD specification is determined by two opposing signals, FGFs from the AER and retinoic acid from the flank mesoderm, regulating gene expression along the PD axis [9,10]. However, no clockwork mechanism had been identified yet.

In their review, Sheeba *et al.* gather arguments in favor of what might be the clock the limb has been expecting for over the 60 last years. The observation that grounds this clock is the cyclic/oscillatory expression of… the *hairy2* gene [11]. The oscillation cycle of *hairy2* is exactly 6 h and two of these *hairy2* cycles correlate with the formation of one bone element (12 h) – but this is a critical issue that is discussed below. The correlation is, however, only true for the distal-most mesenchyme of the limb, as *hairy2* expression is excluded from the anterior area while it is permanent in the posterior region of the limb. Literature supports other interesting parallels between somitic and limb clocks. In terms of the signaling network, gradients of Shh and FGF8 originating from the zone of polarizing activity (ZPA) and AER in the limb, respectively, are akin to the somitic clock scenario wherein Shh and FGF8 gradients arising from the notochord and tail bud correspondingly play an essential role [12]. Markedly, *hairy2* is expressed in the distal limb, in close vicinity of both the ZPA and AER, thereby suggesting that it can be regulated by Shh and FGF gradients. Some papers further propose a GO signal, as *hairy2* expression begins to oscillate at stage HH20, a time when the distal limb becomes sufficiently distanced from the RA source to set up signaling conditions appropriate for this periodic behavior. These are the major points this review addresses in strong conviction, and there is certainly reason to believe that a clock is ticking in limb cells.

However, apart for the cyclic expression of *hairy2* and the analogy between signaling pathways at work in the segmental plate and the limb bud, many links are missing before we can claim that we hold a clockwork mechanism in the limb, which would be responsible for the formation of segmented – if not segmental—bony structures. Quite clearly, there is a clock ticking there, but up to now, no determination front it might control has been described. Thus, we have no link between the clock periodical activity and a mechanism it would control to direct morphogenesis of the digit anlagen.

A major reason why the somitic clock was readily accepted lies into the fact that the dynamics of somite formation was very well documented. Somites are rather simple structures, and deciding when they form is a rather straightforward issue. Thus, demonstrating that expression of *hairy1* was oscillating exactly in phase with the pace of somite formation was a very attractive basis to propose that the two were causally linked and a strong incitation to investigate further. The dynamics of skeletal element formation in the limb is much less documented. First, it is difficult to decide when a digit forms: is it when cartilage first differentiates or when mesenchyme starts condensing to prefigure the aspect of the future skeleton? In the latter case, we cruelly lack markers for these early events. Many attempts have been made to solve this issue, which are reviewed by Sheeba *et al.* With the progress of *in situ* imaging and the identification of new probes (such as *noggin*) to follow the earliest phases of mesenchyme condensation, there is little doubt that the dynamics of limb skeleton formation will be worked out. If this confirms that, at least, part of it is synchronous with the oscillation of *hairy2* expression, we will feel more confident of a causal link between the clock ticking and the bones rising. Working along these lines is therefore a priority.

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